

REMARKS

This Amendment is submitted in reply to the Non-Final Office Action mailed on January 8, 2009. There are no fees due herewith this Amendment. The Commissioner is hereby authorized to charge any fees that may be required or credit any overpayment to Deposit Account No. 02-1818. If such a withdrawal is made, please indicate the Attorney Docket No. 0112701-00737 on the account statement.

Claims 1-26 are pending in this application. Claims 1-8, 19-20 and 22 were previously withdrawn from consideration. In the Office Action, the specification is objected to. Claims 9-15, 17-18 and 21 are rejected under 35 U.S.C. § 112. Claims 9-12, 13-18, 21 and 23-26 are rejected under 35 U.S.C. § 102. In response, Claims 9, 12 and 16 have been amended and Claims 10, 17-18, 21-22 and 25-26 have been canceled without prejudice or disclaimer. The amendments do not add new matter. In view of the amendments and/or for at least the reasons set forth below, Applicants respectfully submit that the rejections should be reconsidered and withdrawn.

In the Office Action, the specification is objected to. Specifically, the Patent Office asserts that the “use of the trademarks Encompress, Promega, Eukit, RNase ZAP, Applied Biosystems, Agilent Technologies, Spectra Fluor Plus F, Eppendorf, Ambion, Q Biogene, Fluka, Molecular Probes, Invitrogen, Amersham Biosciences, Ribogreen have been noted in [the] application. [The trademarks] should be capitalized wherever [they] appear[] and [should] be accompanied by the generic terminology.” See, Office Action, page 3, lines 1-5. According to the Manual of Patent Examining Procedures, Trademarks and Names Used in Trade may be used in patent applications if a) their meanings are established by an accompanying definition which is sufficiently precise and definite to be made a part of a claim, or b) in this country, their meanings are well-known and satisfactorily defined in the literature. Trademarks are defined as a proprietary word, letter, symbol, or device pointing distinctly to the product of one producer. Names Used in Trade are defined as a nonproprietary name by which an article or product is known and called among traders or workers in the art, although it may not be so known by the public, generally. See, MPEP, § 608.01(v). As such, Applicants initially note that the company names identified by the Patent Office in the Office Action need not be designated as trademarks.

These companies include, for example, Promega, Applied Biosystems, Agilent Technologies, Eppendorf, Ambion, QBiogene, Fluka, Invitrogen and Amersham Biosciences.

Further, Applicants have submitted herewith a Substitute Specification and a Marked-Up Version of the Specification. The Substitute Specification does not contain new matter. With respect to the remaining identified terms, which include, Encompress, Eukitt, RNaseZAP, SpectraFluor Plus, Molecular Probes and RiboGreen, Applicants note that the presently submitted Substitute Specification properly identifies these terms as trademarks and includes generic terminology. For at least the above-mentioned reasons, Applicants respectfully request that the objections to the specification be reconsidered and withdrawn.

In the Office Action, Claims 17-18 and 21 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. Specifically, the Patent Office asserts, for example, that “it would be impossible to prevent someone from getting all types of skin disorders through genetic disposition.” See, Office Action, page 4, lines 11-12. In response, Applicants have canceled Claims 17-18 and 21-22 without prejudice or disclaimer. For at least the above-mentioned reasons, Applicants respectfully submit that the rejection of Claims 17-18 and 21 is now rendered moot.

Accordingly, Applicants respectfully submit that the rejection of Claims 17-18 and 21 under 35 U.S.C. § 112, first paragraph be reconsidered and withdrawn.

In the Office Action, Claims 9-15 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Specifically, the Patent Office asserts that in Claim 9, the term “improving” is subject to interpretation and is, thus, indefinite. The Patent Office also asserts that Claims 9-15 are indefinite because it is not clear what is exactly encompassed by “derivatives” of flavanones. See, Office Action, page 6, lines 1-13. In response, Applicants note that independent Claims 9 and 23 have been amended to recite, in part, at least one flavanone compound selected from the group consisting of isosacuranetin, naringin, hesperidin, eriodictyol, poncirin, neoeriocitrin or a derivative of at least one flavanone selected from the group consisting of their aglycone, chalcone, glycosylated or methylated forms and sulfated and glucuronidated forms which are a product of their metabolism in blood, or a mixture thereof.

The amendments do not add new matter. The amendments are supported in the specification at, for example, page 3, lines 19-26.

Applicants submit that the skilled artisan would have immediately appreciate what is meant by the term “improving” in independent Claim 9. For example, “improvement” is defined by Merriam Webster OnLine as “the state of being improved.” “Improved” is defined by Merriam Webster as “to enhance in value or quality.” See, Merriam Webster OnLine, definitions of “improvement” and “improved.” Accordingly, the skilled artisan would understand that “improving” the skin health of a human or animal would comprise “enhancing the value or quality” of the skin of the human or animal. Moreover, the Examples of the specification illustrate that rats fed with a hesperidin supplemented diet showed significantly reduced levels of IL-6 when compared to rats fed a control diet not containing hesperidin. The skilled artisan would immediately appreciate that the results of the test, thus, demonstrate cytoprotective and anti-inflammatory properties of orally administered compositions having flavanones or derivatives thereof. Accordingly, the skin of the rats fed the experimental diet was “improved” with respect to the skin of the rats fed the control diet. As such, Applicants respectfully submit that the skilled artisan would immediately appreciate what is meant by the term “improved,” as used in the present claims, when read in view of the specification.

Applicants also respectfully submit that the skilled artisan would immediately appreciate what is meant by “derivatives” of flavanones when the present claims are read in view of the specification. For example, as discussed above, independent Claims 9 and 23 have both been amended to include, in part, the specific flavanone derivatives listed in the specification at page 3, lines 19-26. Thus, it is very clear that the claims include derivatives of flavanones that are the aglycone, chalcone, glycosylated or methylated forms and sulfated and glucuronidated forms of flavanones, which are a product of their metabolism in blood. Applicants respectfully submit that the skilled artisan would understand that the scope of such a term would not include such basic naturally occurring elements such as carbon and hydrogen, as alleged by the Patent Office. See, Office Action, page 6, lines 10-11. Instead, the skilled artisan would appreciate that the derivatives include the forms as included in the presently amended claims. In view of the amendments, Applicants respectfully submit that the skilled artisan would immediately

appreciate what is meant by the phrase “derivatives” of flavanones when read in view of the specification.

For at least the above-mentioned reasons, Applicants respectfully submit that Claims 9-15 fully comply with the requirements of 35 U.S.C. § 112, second paragraph.

Accordingly, Applicants respectfully request that the rejection of Claims 9-15 under 35 U.S.C. § 112, second paragraph be reconsidered and withdrawn.

In the Office Action, Claims 9-10 and 13-15 are rejected under 35 U.S.C. §102(b) as being anticipated by EP 0 774 249 to Cho et al. (“*Cho*”). Claims 9-10 and 16 are rejected under 35 U.S.C. §102(b) as being anticipated by EP 0 461 827 to Miyake et al. (“*Miyake*”). However, Applicants respectfully submit that *Cho* and *Miyake* are deficient with respect to the present claims.

Currently amended independent Claim 9 recites, in part, methods for improving skin of a human or pet animal comprising the step of orally administering a therapeutically-effective amount of a composition comprising at least one flavanone compound or its derivatives. The amendments do not add new matter. The amendments are supported in the amended specification at, for example, page 4, lines 1-15; page 6, lines 7-9. Applicants have found that the presently claimed methods of administering compositions having at least one flavanone compound or its derivatives or a mixture thereof results in an improved skin health related to a range of skin disorders including, for example, photoprotection, hydration, dryness, firmness, thickness, etc. See, specification, page 3, lines 1-9. In contrast, however, Applicants respectfully submit that *Cho* and *Miyake* fail to disclose each and every element of the present claims.

For example, *Cho* and *Miyake* fail to disclose or suggest methods for improving skin of a human or pet animal comprising the step of orally administering a therapeutically-effective amount of a composition comprising at least one flavanone compound or its derivatives as required, at least in part, by independent Claim 9. Instead, *Cho* is entirely directed toward cosmetic compositions containing combinations of flavanones. See, *Cho*, Abstract. Indeed, *Cho* specifically discloses that the cosmetic compositions are topical compositions. See, *Cho*, page 3, line 6. Similarly, *Miyake* is entirely directed toward topical hair restorer compositions in the form of liquids, gels, emulsions, aerosols, ointments, etc. See, *Miyake*, page 4, lines 32-34.

Accordingly, both *Cho* and *Miyake* are directed entirely toward topical administration of compositions, which is in direct contrast to the presently claimed orally administered compositions. Therefore, since *Cho* and *Miyake* fail to disclose or suggest methods for improving skin of a human or pet animal comprising the step of orally administering a therapeutically-effective amount of a composition comprising at least one flavanone compound or its derivatives, *Cho* and *Miyake* fail to anticipate the present claims.

In the Office Action, Claims 17-18 and 23-26 are rejected under 35 U.S.C. §102(b) as being anticipated by the article Flavanone absorption after naringin, hesperidin, and citrus administration to Ameer et al. ("Ameer"). However, Applicants respectfully submit that *Ameer* is deficient with respect to the present claims.

Applicants initially note that Claims 17-18 have been canceled without prejudice or disclaimer. Thus, the rejection of Claims 17-18 as anticipated by *Ameer* is now rendered moot. Further, independent Claim 23 recites, in part, methods for treating a disorder selected from the group consisting of disorders of the skin, hair and coat comprising the step of orally administering to an individual having a disorder of the skin, hair or coat a therapeutically-effective amount of a composition. As discussed above, Applicants have found that the presently claimed methods of administering compositions having at least one flavanone compound or its derivatives or a mixture thereof results in an improved skin health related to a range of skin disorders including, for example, photoprotection, hydration, dryness, firmness, thickness, etc. See, specification, page 3, lines 1-9. In contrast, however, Applicants respectfully submit that *Ameer* fails to disclose each and every element of the present claims.

Ameer fails to disclose or suggest methods for treating a disorder selected from the group consisting of disorders of the skin, hair and coat comprising the step of orally administering to an individual having a disorder of the skin, hair or coat a therapeutically-effective amount of a composition as required, in part, by the present claims. Instead, *Ameer* is entirely directed toward the absorption of citrus flavanoids in the GI tract. *Ameer* specifically discloses administering single-doses of pure compound and multiple-doses of citrus fruit and citrus fruit juice to a subject that was "healthy, within 10% of his ideal body weight, and was taking no medication. He did not smoke tobacco or drink alcohol." Further, three additional adults

participated in the study having ages ranging between 25 and 87. See, *Ameer*, Methods, first paragraph.

The Patent Office alleges that the “patient is the same [in the present claims and *Ameer*] because every person has skin” and “[t]hus, on the administration of hesperidin to any patient, a prevent of skin disorder would have had to occur if applicant’s invention function[ed] as claimed.” See, Office Action, page 7, line 21-page 8, line 2. However, Applicants first note that independent Claim 23 does not recite “preventing” skin disorders, but rather recites, in part, methods for treating a disorder selected from the group consisting of disorders of the skin, hair and coat comprising the step of orally administering to an individual having a disorder of the skin, hair or coat a therapeutically-effective amount of a composition. As such, independent Claim 23 requires, in part, administration of compositions to an individual having a skin, hair or coat disorder. *Ameer* fails to disclose or suggest administering the compositions to individuals having any disorders, let alone disorders of the skin, hair or coat as is required, in part, by the present claims. For at least the reasons set forth above, *Ameer* cannot disclose or suggest every element of the present claims and, therefore, fails to anticipate the present claims.

In the Office Action, Claims 9-12, 14-15, 17-18, 21 and 23-24 are rejected under 35 U.S.C. §102(b) as being anticipated by U.S. Patent No. 4,049,798 to Bottomley (“*Bottomley*”). However, Applicants respectfully submit that *Bottomley* is deficient with respect to the present claims.

Applicants initially note that Claims 17-18 have been canceled without prejudice or disclaimer. Thus, the rejection of Claims 17-18 as anticipated by *Bottomley* is now rendered moot. Further, currently amended independent Claims 9 and 23 have been amended to recite, in part, at least one flavanone compound selected from the group consisting of isosacuranetin, naringin, hesperidin, eriodictyol, poncirin, neoeriocitrin or a derivative of at least one flavanone selected from the group consisting of their aglycone, chalcone, glycosylated or methylated forms and sulfated and glucuronidated forms which are a product of their metabolism in blood, or a mixture thereof. The amendments do not add new matter. The amendments are supported in the specification at, for example, page 3, lines 19-26. In contrast, however, Applicants respectfully submit that *Bottomley* fails to disclose each and every element of the present claims.

Bottomley fails to disclose or suggest compositions comprising at least one flavanone compound selected from the group consisting of isosacuranetin, naringin, hesperidin, eriodictyol, poncirin, neoeriocitrin or a derivative of at least one flavanone selected from the group consisting of their aglycone, chalcone, glycosylated or methylated forms and sulfated and glucuronidated forms which are a product of their metabolism in blood, or a mixture thereof as required, in part, by the present claims. Instead, *Bottomley* is entirely directed toward the treatment of herpes simplex comprising administration of a mixture comprising Vitamin C and Vitamin P. See, *Bottomley*, Abstract. Indeed, *Bottomley* fails to disclose or suggest any specific bio-flavanoids anywhere in the disclosure. Because *Bottomley* fails to disclose or even suggest the specific flavanones or derivatives thereof as presently claimed, *Bottomley* fails to anticipate the present invention.

Further, anticipation is a factual determination that “requires the presence in a single prior art disclosure of each and every element of a claimed invention.” *Lewmar Marine, Inc. v. Barient, Inc.*, 827 F.2d 744, 747 (Fed. Cir. 1987) (emphasis added). Federal Circuit decisions have repeatedly emphasized the notion that anticipation cannot be found where less than all elements of a claimed invention are set forth in a reference. See, e.g., *Transclean Corp. v. Bridgewood Services, Inc.*, 290 F.3d 1364, 1370 (Fed. Cir. 2002). As such, a reference must clearly disclose each and every limitation of the claimed invention before anticipation may be found. Because the cited references fail to disclose each and every element of the present claims, the cited references fail to anticipate the present claims.

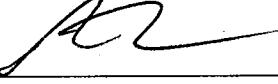
Accordingly, Applicants respectfully submit that the anticipation rejections with respect to Claims 9-12, 13-18, 21 and 23-26 be reconsidered and withdrawn.

For the foregoing reasons, Applicants respectfully request reconsideration of the above-identified patent application and earnestly solicit an early allowance of same. In the event there remains any impediment to allowance of the claims which could be clarified in a telephonic interview, the Examiner is respectfully requested to initiate such an interview with the undersigned.

Respectfully submitted,

K&L GATES LLP

BY



Robert M. Barrett
Reg. No. 30,142
Customer No. 29157
Phone No. 312-807-4204

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TITLE

**"COMPOSITION FOR IMPROVING SKIN, HAIR AND COAT HEALTH
CONTAINING FLAVANONES"**

[0001] The present invention pertains to a composition for preventing, decreasing and/or treating skin and hair/coat disorders or damages, such as is effected by inflammatory reactions, environmental factors, ageing or cancer. In particular, the present invention relates to the use of flavanones compounds or their derivatives in nutritional, cosmetic or pharmaceutical compositions for improvement of human or pet animal skin and coat conditions.

BACKGROUND OF THE INVENTION

[0002] The most prominent epithelial tissue in living beings is the skin, which represents the largest organ in the organism. The system of skin integument, which comprises the epidermis, dermis and the stratum corneum, correlates with those of internal organs and concurrently interacts with the surroundings. Being the interface between the environment and organism itself, the skin is heavily influenced by external factors and also variable parameters of the organism's inner system. The skin's regulative mechanisms need, therefore, always be active to induce systemic changes necessary to maintain normal pathological events concerning skin integument morphology and activities.

[0003] A great deal of processes assuring the adequate consumption of increased affluence of energetic and plastic substances according to the skin's needs become guarantors of morphological and functional stability of skin structures. So, the state of integuments determines the realization of metabolic processes necessary for skin cell viability and activity leading to the presence of healthy skin peculiarities such as barrier function, elasticity, turgor properties, humidity, pigmentation etc..etc.

[0004] During the lifetime of a living being different signs, characteristic of ageing, appear on the skin or hair, with the principal clinical signs being the appearance of

fine lines and deep wrinkles which increase or are accentuated with age, loss of hair, reduced hair density, glossiness, color, oilness, fiber diameter, etc....etc.

[0005] These signs of ageing are even promoted by exposure of the skin and hair to exogenous influences, such as e.g.e.g., UV-radiation, pollutants, free radicals or chemical substances.

[0006] In the art several means have been proposed to prevent destructive effects of environment or ageing on skin epithelial cells. For example, means to prevent skin deterioration or ageing is to provide compounds scavenging free radicals. In this respect EP 0 761 214 discloses singlet oxygen quenchers comprising aniline derivatives and difurfuryl amine derivatives, which are reported to reduce the oxidative stress to the skin.

[0007] Although there is a great diversity of active compounds for ameliorating skin and hair or coat health, there still exists a need in the art to provide new active compounds. In particular, an object of the present invention is to provide compositions that may be used over a long period of time by humans or pets, and susceptible to be provided in the form of a nutritional supplement, for example a nutritional composition.

SUMMARY OF THE INVENTION

[0008] In a first aspect, the present invention relates to a nutritional, cosmetic or pharmaceutical composition for human or pets, which contains as active compound, at least one flavanone compound or its derivatives, or a mixture thereof, in an efficient amount to prevent, treat or alleviate skin, hair and/or coat disorders and ameliorate skin, hair and coat health.

[0009] In another aspect, the invention provides the use of at least one flavanone or its derivatives or mixtures thereof, as active compound in the preparation of a nutritional, cosmetic or pharmaceutical composition intended for preventing or treating skin, hair and/or coat disorders, thus ameliorating skin health conditions in humans or pets.

[0010] The composition according to the present invention may be in the form of a complete nutritional formula, a dietary supplement to be orally administered to a human or an animal, or a compound for pharmaceutical use.

[0011] Administering to a human or pet animal, a food composition as described above, results in an improved skin health, e.g.e.g., on photoprotection, hydration, dryness, firmness, thickness, elasticity, oilness, regular pigmentation, immunity or hair and coat health, e.g.e.g., improving hair and coat gloss, hair density, color, oilnessoilness, ameliorating hair fibre—fiber diameter, sebum production, glossynes glossiness and preventing hair and coat loss. Also, the composition according to the present invention is administered to a human or an animal, for ameliorating antioxidant status, barrier function, to prevent or modulate oxidative status, sebum production or composition, or to reduce signs of ageing. It also helps to reduce risks of cancer or inflammation.

[0012] Additional features and advantages are described herein, and will be apparent from the following Detailed Description and the figures.

BRIEF DESCRIPTION OF THE FIGURES

[0013] Fig. 1: HaCat cells were incubated with 10 µM hesperetin (hp, red bars) or low 10 µM hesperetin-7-O-glucuronide (hp-7-O-gluc, yellow bars) or equal amounts of DMSO as a control (blue bars) and treated with or without menadione for additional 5 h. The supernatant was analyzed for lactate dehydrogenase (LDH) activity and results were expressed relative to cells which were lysed with Triton X-100® before analysis (100 % death).

[0014] Fig. 2: Chart representing the experimental set-up of the hesperidin growth trial.

[0015] Fig. 3: Histopathological analysis of rat skin supplemented with hesperidin. 6 µm paraffin sections were de-waxed, stained with hematoxylin/eosin and mounted. Representative images in two magnifications are shown for the control group (A and D) and the groups supplemented with hesperidin (0.1%: B and E, 0.5% C and F).

[0016] **Fig. 4:** Real-time PCR analysis of total RNA isolated from rat skin fed either a control diet (ctrl) or a hesperidin-supplemented diet (0.1% Hp, 0.5% Hp) for the expression of CD1d1 and interleukin 6 (IL-6). Samples were analyzed in 3 pools containing 4 rats each and obtained Ct values are shown for CD1d1 in A and IL-6 in B. Dots represent averages of technical triplicates, bars the total average per group. Fold changes in relative expression of the supplementation compared to control diet and relative to a housekeeping gene are shown in C. The control diet was set to 1 fold and is represented by a thick line. Confident intervals were calculated using ANOVA.

DETAILED DESCRIPTION OF THE INVENTION

[0017] According to the first object, the invention provides a nutritional, cosmetic or pharmaceutical composition for oral administration for human or pets, which contains as active compound, at least one flavanone compound or its derivatives, or a mixture thereof, in an efficient amount to prevent, treat or alleviate skin, hair and/or coat disorders or damages and thus ameliorate skin, hair and coat health.

[0018] The flavanone compounds of interest are natural glycosides that can be found principally in fruits from the genus Citrus, such as orange, lemon, bitter orange, grapefruit, for example or in a lesser extend in other vegetables. They are present in majority in the peel of the fruit, but also in large amounts in the pulp and thus also in citrus fruit juice. The compounds according to the present invention may be isosacuranetin, naringin, hesperidin, or eriodictyol, poncirin, neoeriocitrin, for example, and their derivatives selected from their aglycone forms, chalcone forms, glycosylated forms or methylated forms. Also, their sulfated or glucuronidated forms which are found as product of metabolism in blood are used.

[0019] In a last aspect, derivatives may be obtained by several processes known in the art, such as enzymatic treatments. For example, glucose-7-hesperetin is prepared by rhamnosidase or hesperidinase treatment.

[0020] The flavanone compound or derivatives according to the invention may be included in any composition suitable for administering the substance to an individual,

in particular a food composition, a cosmetic composition or a pharmaceutical composition.

[0021] In a prefered-preferred embodiment, a food composition for human consumption is prepared. This composition may be a nutritional complete formula, a dairy product, a chilled or shelf stable beverage, soup, a dietary supplement, a meal replacement, and a nutritional bar or a confectionery. The composition may be selected from the group consisting of milk, or fermented milk products, such as e.g. yogurt, curd, cheese, milk based fermented products, ice-creams, milk based powders, infant formulae, cereal products and fermented cereal based products, beverages, mineral water, chocolate or pet food containing at least a flavanone compound or one of its derivatives. The nutritional supplement for oral administration may be in capsules, soft capsules, tablets, pastes or pastilles, gums, or drinkable solutions or emulsions. Methods for preparing them are common knowledge.

[0022] As described above, flavanones compounds are found naturally in Citrus fruits, in particular in oranges, lemons and grapefruit, in their peel or pulp. Accordingly, in a first aspect, the nutritional composition may be in the form of a juice of such fruits or in the form of a concentrate. Thus, the nutritional composition may be in the form of any food product, in particular any beverage, citrus juice or any other extract from peel or pulp of citrus fits.

[0023] In another embodiment, a usual food product may be enriched with the flavanones, preferably in the form of citrus extract. For example, a fermented milk, a yoghurt, a fresh cheese, a renneted milk, a confectionery bar, breakfast cereal flakes or bars, drinks, milk powders, soy-based products, non-milk fermented products or nutritional supplements for clinical nutrition. In particular, a process for preparing an extract enriched if flavanones, in particular hesperidin, from orange and lemon is described in US N02,400,693No. 2,400,693 and US No. 2,442,110, respectively.

[0024] According to a further aspect, flavanones compounds to be included in the specification may be synthetically produced.

[0025] A nutritional composition according to the present invention may comprise-comprise the flavanone compounds, its derivatives or mixtures thereof in an

amount adapted to a daily oral administration, and of from about 0.01 mg to 1g, preferably from about 0.1 mg to 800 mg, more preferably from 10 mg to 800 mg of the aglycone equivalent of the flavanone compound.

[0026] The flavanones according to the invention may be used either alone or in association with other active compounds such as vitamin C, vitamin E (tocopherols and tocotrienols), carotenoids (carotenes, lycopene, lutein, zeaxanthine, beta-cryptoxanthine, etc..etc.) ubiquinones (e.g.CoQ10e.g., CoQ10), catechins (e.g.e.g., epigallocatechin gallate), coffee extracts containing polyphenols and/or diterpenes (e.g.e.g., kawheol and cafestol), extracts of chicory, ginkgo biloba extracts, grape or grape seed extracts rich in proanthocyanidins, spice extracts (e.g.e.g., rosemary), soy extracts containing isoflavones and related phytoestrogens and other sources of flavonoids with antioxidant activity, fatty acids, e.g.e.g., n-3 fatty acids, prebiotic fibers, probiotic microorganisms, taurine, resveratrol, aminoacids, selenium and precursors of glutathione, for example.

[0027] In another embodiment, a pharmaceutical compositions can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease, as described herein under, in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as "a therapeutically effective dose". Amounts effective for this will depend on the severity of the disease and the weight and general state of the patient.

[0028] In prophylactic applications, compositions according to the invention are administered to a patient susceptible to or otherwise at risk of a particular disease. Such an amount is defined to be "a prophylactic effective dose". In this use, the precise amounts again depend on the patient's state of health and weight. Preferably, for humans the pharmaceutical composition according to the present invention comprises an amount of flavanone compounds, its derivatives or mixture thereof as described above, for a daily administration, from about 0.01 mg to 500 mg. When administered daily to pets, the composition may comprise from 1 mg ~~et~~to 500 mg of the aglycone equivalent of flavanone compounds.

[0029] The compounds of the invention are preferably administered with a pharmaceutical acceptable carrier, the nature of the carrier differing with the mode of administration, for example parenteral, intravenous, oral and topical (including ophthalmic) routes.

[0030] It will be appreciated that the skilled person will, based on his own knowledge select the appropriate components and galenic form to target the active compound to the skin or hair taking into account the route of administration which may be by way of injection, topical application, intranasal administration, administration by implanted or transdermal sustained release systems, and the like.

[0031] The objective substance may also be formulated in a cosmetic product, such as lotions, shampoos, creams, sun-screens, after-sun creams, sun-blocker, anti-ageing creams and/or ointments. It will be appreciated that the present cosmetic products will contain a mixture of different ingredients known to the skilled person, ensuring a fast penetration of the objective substance into the skin and preventing degradation thereof during storage.

[0032] It will be understood that the concept of the present invention may likewise be applied as an adjuvant therapy assisting in presently used medications. Since the compounds of the present invention may easily be administered together with food material special clinical food may be applied containing a high amount of the objective substances. It will be clear that on reading the present specification together with the appending claims the skilled person will envisage a variety of different alternatives to the specific embodiments mentioned herein.

[0033] In principle, the compounds according to the present invention may be used for the treatment and/ or prevention of damages in the skin which are produced by a stress situation e.g.e.g., by means of a chemical, biological or a physical stress, e.g.e.g., by exposure to oxidants or carcinogens, exposure to bacteria, viruses, fungi, lipids derived from surrounding cells and/or microbes, or exposure to UV-irradiation.

[0034] Consequently, the substances and/or compositions according to the present invention may be utilized for treating and or preventing damages of the skin, in particular actinic and ageing damages of the skin such as dryness, actinic keratoses,

irregular pigmentation (notably comprising freckling, lentigines, guttate hypomelanosis and persistent hyperpigmentation), wrinkling (notably comprising fine surface lines and deep furrows), stellate pseudoscars, elastosis, inelasticity, telangiectasia, venous lakes, purpura, comedones, sebaceous hyperplasia, acrochordon, cherry angiogema, seborrhea keratosis, lentigo, basal cell carcinoma and squamous cell carcinoma, skin burning and/or blistering, epidermal hyperplasia, inflammation, immune suppression, and cancer, e.g., non-melanoma and melanoma skin cancers. They have also particular benefits on hair and coat, such as an improved hair or coat density, fiber diameter, color, oiliness, glossyness, sebum production and a helps to prevent hair or coat loss.

[0035] The effect of a food supplementation in flavanones compounds or its derivatives according to the present invention, on skin of humans or pets, can be measured by using conventional methods including minimal erythema1 dose (MED), colorimetry, transepidermal water loss, DNA repair (e.g., p. 53), measure of interleukines and proteoglycans production, or collagenase activity, barrier function or cell renewal.

[0036] The following examples illustrate the invention in more detail without restricting the same thereto. They are proceeded by a brief description of the Figures.

[0037] **Fig. 1:** HaCat cells were incubated with 10 μ M hesperetin (hp, red bars) or low 10 μ M hesperetin 7-O-glucuronide (hp 7-O-gluc, yellow bars) or equal amounts of DMSO as a control (blue bars) and treated with or without menadione for additional 5h. The supernatant was analyzed for lactate dehydrogenase (LDH) activity and results were expressed relative to cells which were lysed with trition XI00 before analysis (100% death).

[0038] **Fig. 2:** Chart representing the experimental set up of the hesperidin growth trial.

[0039] **Fig. 3:** Histopathological analysis of rat skin supplemented with hesperidin. 6 μ m paraffin sections were de-waxed, stained with hematoxylin/eosin and

mounted. Representative images in two magnifications are shown for the control group (A and D) and the groups supplemented with hesperidin (0.1%: B and E, 0.5% C and F).

[0040] **Fig. 4:** Real time PCR analysis of total RNA isolated from rat skin fed either a control diet (ctrl) or a hesperidin supplemented diet (0.1% Hp, 0.5% Hp) for the expression of CD1d1 and interleukin 6 (IL 6). Samples were analyzed in 3 pools containing 4 rats each and obtained Ct values are shown for CD1d1 in A and IL 6 in B. Dots represent averages of technical triplicates, bars the total average per group. Fold changes in relative expression of the supplementation compared to control diet and relative to a housekeeping gene are shown in C. The control diet was set to 1 fold and is represented by a thick line. Confident intervals were calculated using ANOVA.

[0036] It should be understood that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art. Such changes and modifications can be made without departing from the spirit and scope of the present subject matter and without diminishing its intended advantages. It is therefore intended that such changes and modifications be covered by the appended claims.

[0037] The following examples illustrate the invention in more detail without restricting the same thereto.

[0038] **Example 1: mineral water supplemented with flavanone**

[0039] A mineral water is prepared by adding hesperitin-7-glucose, in an amount of 0.01 mg to 200 mg per liter, estimating that the average consumption is of about 1 liter per day.

[0040] **Example 2: Cosmetic for oral administration**

[0041] A composition in the form of a hard capsule has the following formulation:

Compound	mg per capsule
Hesperidine (hesperetin equivalent)	250
Excipient for the core	
Cellulose microcrystalline	70
Encompress™ <u>(dicalcium phosphate dihydrate)</u>	60
Stéarate de Magnesium	3
Silice colloidale anhydre	1
Coating agent	
Gum-lac	5
Talc	61
Sucrose	250
polyvidone	6
titanium dioxide	0.3
coloring agent	5

[0042] The composition can administered to the individual in an amount of 2 to 3 capsules daily.

[0043] **Example 3: Canned Pet food and supplement**

[0044] A mixture is prepared from 73 % of poultry carcass, pig lungs and beef liver (ground), 16 % of wheat flour, 7 % of water, 2 % of dyes, flavours, vitamins, and inorganic salts. This mixture is emulsified at 12°C and extruded in the form of a pudding which is then cooked at a temperature of 90°C. It is cooled to 30°C and cut in chunks. 45 % of the chunks are mixed with 55 % of a sauce prepared from 98 % of water, 1 % of dye and 1%1% of guar gum. Tinplate cans are filled and sterilized at 125°C for 40 min.

[0045] As a supplement to be mixed with the pet-food before serving, additional packaging in sachet form with 50 mg of hesperetin equivalent, in the form of Citrus extract is provided. This is supplied as a supplement with removably attached to the can, together with feeding directions.

[0046] **Example 4: Functional food**

[0047] A food supplement was prepared by mixing or blending fructooligosaccharide with inulin in the proportions by weight of about ~~70%70 %~~ fructooligosaccharide to about ~~30%30 %~~ inulin and adding 500 mg of hesperetin equivalent. The resulting prebiotic mixture may be added or blended with any suitable carrier, for example a fermented milk, a yogurt, a fresh cheese, a renneted milk, a confectionery bar, breakfast cereal flakes or bars, a drink, milk powder, soy-based product, non-milk fermented product or a nutritional supplement for clinical nutrition.

[0048] **Example 5**

[0049] **Material and Methods**

[0050] **Cytotoxicity Assay**

[0051] Human immortalized keratinocytes (HaCaT) were incubated with $10\mu\text{M}$ hesperetin, ~~$10\mu\text{M}$~~ $10\mu\text{M}$ hesperetin-7-O-glucuronide or equal amounts of DMSO as a negative control for ~~16h~~ 16 h and ~~4h~~ 1 h before challenge. Cells were then treated with ~~$100\mu\text{M}$~~ $100\mu\text{M}$ menadione, a xenobiotic which generates reactive oxygen species intracellularly. Non-menadione treated cells were used as a positive control. After ~~5h~~ 5 h the supernatant was analysed for lactate dehydrogenase (LDH) activity as a measure for cell death using the CytoTox 96 non-radioactive cytotoxicity assay (Promega, USA).

[0052] **Skin Samples**

[0053] Rat skin biopsies were obtained from the Heperidin growth trial-(Fig.2) (Fig. 2). Dorsal skin was excised, one part was fixed in 4%4 % PFA and paraffin embedded, one part was cryo-preserved and another part was immediately frozen in liquid nitrogen.

[0054] **Histology**

[0055] *Paraffin sections*

[0056] Rat skin was dissected and fixed for 4 days in 4%4 % paraformaldehyde in PBS (pH 7.4) at 4°C and embedded in paraffin using a Leica MicrosystemeMicrosystem embedding apparatus. The tissues were washed in PBS and saline (0.9%0.9 % NaCl) and dehydrated by passing them through saline solutions with increasing ethanol concentrations: 30 min each in 30%, 50%, 70%, 90%, 99%, 100%30 %, 50 %, 70 %, 90 %, 99 %, 100% and an additional hour in 100%100 %. Tissue samples were incubated twice for 30 min in xylene, followed by 2-3 h and 3 h incubations in paraffin wax at 60°C. 6 µm thick paraffin sections were cut using a Leica Microtome. Sections were de-waxed 5 min in xylene and dehydrated by passing them through a series of solutions with decreasing ethanol concentrations: 1 min each in 100%, 96%, 90%, 80%, 70%100 %, 96 %, 90 %, 80 %, 70%, and 50%50 % ethanol. Finally, they were transferred into distilled water and stained.

[0057] *Hematoxylin/eosin staining*

[0058] Rehydrated sections were stained for 45 sec in Mayer's hematoxylin solution, rinsed with the following series of solution for 1 min each: distilled water, tap water, distilled water and 70%70 % ethanol. After staining 10 sec in eosin solution (1%1 % (v/v) in 90%90 % ethanol) sections were rinsed in 90%90 % and 100%100 % ethanol. Following two 10 min incubations in xylene, coverslips were mounted with EukitEukitt® quick-hardening mounting medium and air-dried for 2 h at room temperature.

[0059] **RNA Methods**

[0060] **General directions for working with RNA**

[0061] For experiments with RNA, sterile pastie plastic or baked glass vessels (180°C for at least 8h8 h) have been used. All surfaces were cleaned with RNase ZAP® decontamination solution prior use, including pipetmen, and aerosol resistant tips were used only.

[0062] ***Equipment***

[0063] ABI PRISM® 7000HT Sequence Detection System, Applied Biosystems, USA

[0064] ABI PRISM® 7000 RT-PCR software, Applied Biosystems, USA

[0065] PCR Cycler, e.g.e.g., PTC-100™ Programmable Thermal Controller, MJ Research Inc., USA

[0066] Agilent 2100 bioanalyzer, Agilent Technologies, USA

[0067] Fluorescence Plate Reader, e.g.e.g., Spectra FluorSpectraFLUOR® Plus F 129005, Tecan, USA

[0068] Multifuge 3S, Heraeus with special buckets for MFC centrifugation, Kendro Laboratory Products, Switzerland

[0069] Cooling Centrifuge, e.g.e.g., Centrifuge 5417R, Eppendorf, Germany

[0070] ***Reagents***

[0071] Totally RNA ToTally RNA™ Kit (Art. No. 1910), Ambion, USA

[0072] Lysing Matrix D (Art. No. 6913 – 100), Q BIogene, France

[0073] RNA 6000 Nano Assay (Art. No.5065-4475No. 5065-4475 and 5065-4476), Agilent Technologies, USA

[0074] Assays-on-demand (20x stock, Applied Biosystems, USA)

[0075] RNase ZAP® (Art. No. 9780), Ambion, USA

- [0076] Nuclease-free water (ddH₂O, Art. No. 9939), Ambion, USA
- [0077] Milli-Q filtered water (0.22 μM, ddH₂O)
- [0078] Ethanol, GR for analysis (Art. No. 02860), Fluka
- [0079] Dulbecco's phosphate buffered saline (PBS, Art. No. D8537), Sigma, USA
- [0080] β-Mercaptoethanol (Art. No. M7522), Sigma, USA
- [0081] RiboGreen® RNA Quantitation Kit (Art. No. R-11490), Molecular Probes, USA
- [0082] SUPERase-In™ RNase Inhibitor (20U/μl, Art. No. 2694), Ambion, USA
- [0083] SuperScript™ II RNase H⁻ reverse transcriptase (200U/μl, Art. No. 18064-014), Invitrogen, USA
- [0084] First-strand buffer (5x): 250 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1% NP-40, 50% glycerol, included with SuperScript™ II RNase H⁻ reverse transcriptase
- [0085] Dithiothreitol (DTT, 1 mM), included with SuperScript™ II RNase H⁻ reverse transcriptase
- [0086] 2'-Deoxyadenosin-5'-triphosphate (dATP, 100 mM), Art. No. 272050), Amersham Biosciences, England
- [0087] 2'-Deoxycytidine-5'-triphosphate (dCTP, 100 mM), Art. No. 272060), Amersham Biosciences, England
- [0088] 2'-Deoxyguanosine-5'-triphosphate (dGTP, 100 mM), Art. No. 272070), Amersham Biosciences, England
- [0089] 2'-Deoxythymidin-5'-triphosphate (dTTP, 100 mM), Art. No. 272080), Amersham Biosciences, England
- [0090] pd(N)₆ Random hexamer (Art. No. 27-2166-01), Amersham Biosciences, England
- [0091] TaqMan® Universal PCR Master Mix (Art. No. PN4304437), Applied Biosystems, USA

Gene name	Gene symbol	Reference sequence	Assay ID
interleukin 6	Il6	NM_012589	Rn00561420_m1
CD1d1 antigen	Cd1d1	NM_017079	Rn_00567162_m1
proliferating cell nuclear antigen	Pcna	NM_022381	Rn00574296_g1
glyceraldehydes-3-phosphate dehydrogenase	Gapd	NM_017008	Rn99999916_s1

[0092] **Tab. 1:** Assays-on-demand used (Assay ID), including Gene names, genes symbols and reference sequences.

[0093] *RNA extraction*

[0094] Skin samples were homogenized with Lysing Matrix D, total RNAs were extracted using the Totally RNA ToTally RNA™ Kit following the manufacturer's instructions. RNA was eluted with 40±140 µl of nuclease-free water.

[0095] *RNA quantification*

[0096] The quantification was performed using the Ribogreen® RNA quantitation Kit on 96-well plates and a fluorescence microplate reader according to the manual. Measurements were done in duplicate. The samples were diluted either 1:680 or 1:3400 in a final volume of 100 µl 1xTE buffer. Dilutions of the ribosomal RNA in a concentration of 1, 0.5, 0.1, 0.02, 0 µg/ml were used as standards. Integrity of 1±1 µl RNA was controlled using RNA 6000 Nano Assay.

[0097] *Reverse transcription*

[0098] All manipulations were done on ice. 2±2 µl pd(N)₆ random hexamers and 1±1 µl dNTP (10mM/10 mM) were added to 2±2 µg RNA in nuclease-free water in a final volume of 12±12 µl. After 5min5 min incubation at 65°C, samples were immediately placed on ice and quickly centrifuged. Then, 4±4 µl of 5X first strand

buffer, 2 μ l2 μ l of dithiothreitol, 1 μ l1 μ l of RNase inhibitor and 1 μ l1 μ l of reverse transcriptase SuperScript™ II RNase H- were added (final volume 20 μ l20 μ l). The reverse transcription reaction was performed in a PCR cycler using the following temperature program: activation of the enzyme: 10min10 min at 25°C; reverse transcription reaction: 60min60 min at 42°C; inhibition of the enzyme: 20min20 min at 70°C. The sample was then kept in the freezer at - 20°C until further use.

[0099] *Real-time polymerase chain reaction*

[0100] The real-time PCR was performed according to the TaqMan® method in 96 well plates (96WP) using assays-on-demand primer and probes. Analysis was done in triplicate using a master mix (3.5x) which contained 43.7 μ l43.7 μ l TaqMan® 2x Universal PCR master mix, 4.4 μ l4.4 μ l assays-on-demand primers and probes, 21.9 μ l21.9 μ l μ l nuclease-free water and 17.5 μ l17.5 μ l cDNA (87.5ng = 25ng per replicate). Triplicates of 25 μ l25 μ l master mix were loaded on a 96 well ABI PRISM® reaction plate, covered with a transparent optical adhesive cover and centrifuged three times at 2000rpm2000 rpm for 1min1 min or until all air-bubbles had been removed. The PCR reaction was then performed in the ABI PRISM® 7000 Sequence Detection System using the following temperature program: activation of the enzyme: 2min2 min at 50°C; denaturation: 10min10 min at 95°C and 40 cycles target amplification: 15sec15 sec annealing at 95°C and 1min1 min extension at 60°C. The analysis of the amplification plots was done using the ABI PRISM® software Baseline adjustments were done individually (Il6: 15-25, Cdldl: 10-20, PcnA: 15-25; Gapd: 6-15), whereas thresholds were set manually at 0.2 for all primers. The resulting Ct values were exported into Microsoft Excel for further analysis.

[0101] *Statistical analysis*

[0102] Data were analysed by ANOVA.

[0103] **Results and Discussion**

[0104] In vitro experiments using immortalized keratinocytes (HaCat) demonstrated that treatment with hesperetin (hp) and hesperetin-7-O-glucuronide (hp-7-O-gluc) is reducing cell death under normal culture conditions. The protective effect of hp and hp-7-O-gluc was even more pronounced in cells challenged with menadione, a xenobiotic which increases intracellular levels of reactive oxygen species (ROS). Moreover, hp-7-O-gluc, the main metabolite of hesperidin in blood, seems to be more potent compared to hp, the aglycone (Fig. 1).

[0105] The protective effect of hesperidin was further investigated in an animal interventional trial using growing female wistar rats. After weaning, rats were randomized in 3 groups with 12 animals each and supplemented with either a control diet, or a hesperidin supplemented diet using two different doses (0.1% and 0.5%). At the age of 12 weeks rats were sacrificed and skin tissue was used for skin histology and mRNA analysis (Fig. 2). Histopathological analysis of the skin revealed a reduced number of inflammatory cells in animals fed the hesperidin diet. Representative images are shown in Fig. 3 (3A+D (control) vs. 3B+E (0.1% hesperidin) vs. 3C+F (hesperidin)). These histological observations could be confirmed at the mRNA level. ~~Rat~~Rats fed 0.5% hesperidin showed significantly reduced levels of IL-6, an inflammatory cytokine (Fig. 4A+C). In addition CD1d1 mRNA levels were significantly decreased in both groups supplemented with hesperidin (Fig. 4B+C).

[0106] These data clearly demonstrate cytoprotective and anti-inflammatory properties of orally administrated hesperidin for skin.

ABSTRACT OF THE DISCLOSURE

The present invention pertains to a composition for preventing, decreasing and/or treating skin and hair/coat disorders, such as is effected by inflammatory reactions, environmental factors, ageing or cancer. In particular, the present invention relates to the use of flavanones compounds or their derivatives in nutritional, cosmetic or pharmaceutical compositions for improvement of human or pet animal skin and coat conditions.